

Physiological characterization of *Streptococcus bovis* mutants that can resist 2-deoxyglucose-induced lysis

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***Streptococcus bovis* JB1 does not normally lyse, but stationary phase lysis can be induced by including 2-deoxyglucose (2DG) in the growth medium. Isolates deficient in glucose/2DG phosphotransferase activity (PTS⁻) also lysed when 2DG was present (Lys⁺) and this result indicated that 2DG phosphorylation via the PTS was not an obligate requirement for 2DG-induced lysis. Cells and cell walls from 2DG-grown cultures lysed faster when proteinase K was added, but glucose-grown cultures and cell walls were not affected. A lipoteichoic acid (LTA) extract (aqueous phase from hot phenol treatment) from glucose-grown cells inhibited the lysis of 2DG-grown cultures, but a similar extract prepared from 2DG-grown cells was without effect. Thin-layer chromatography and differential staining indicated that wild-type and Lys⁺ PTS⁻ cells incorporated 2DG into LTA, but lysis-resistant cultures (Lys⁻ PTS⁺ and Lys⁻ PTS⁻) did not. LTA from lysis-resistant (Lys⁻ PTS⁺ and Lys⁻ PTS⁻) cells grown with glucose and 2DG also prevented 2DG-dependent lysis of the wild-type. LTA could not inhibit degradation of cell walls isolated from 2DG-grown cultures, but LTA inhibited the lysis of *Micrococcus lysodeikticus* (*Micrococcus luteus*) cells that were exposed to supernatants from 2DG-grown *S. bovis* cultures. Group D streptococci (including *S. bovis*) normally have an α -1,2 linked glucose disaccharide (kajibiose) in their LTA, but kojibiose cannot be synthesized from 2DG. This observation suggested that the kojibiose moiety of LTA was involved in autolysin inactivation. Wild-type *S. bovis* had ATP- as well as PEP-dependent mechanisms of 2DG phosphorylation and one lysis-resistant phenotype (Lys⁻ PTS⁻) had reduced levels of both activities. However, the Lys⁻ PTS⁺ phenotype was still able to phosphorylate 2DG via ATP and PEP and this result indicated that some other step of 2DG incorporation into LTA was being inhibited. Based on these results, growth in the presence of 2DG appears to prevent synthesis of normal LTA, which is involved in the regulation of autolytic enzymes.**

Keywords: *Streptococcus bovis*, 2-deoxyglucose, cell lysis, lipoteichoic acid

INTRODUCTION

Streptococcus bovis is a facultative anaerobe that is normally found in the rumen of cattle and the colon of simple stomached animals. In the rumen, *S. bovis* overgrowth is associated with a decline in rumen pH, indigestion and even death of the animal (Hungate *et al.*, 1952). *S. bovis* can also infect human tissues and has

been associated with general bacteraemia, heart valve infections and deterioration of the colon wall during colon cancer (Kupferwasser *et al.*, 1988; Zarkin *et al.*, 1990).

Previous work indicated that *S. bovis* lysis could be triggered by the non-metabolizable glucose analogue, 2-deoxyglucose (2DG) (Russell & Wells, 1997). 2DG-induced lysis was only observed if it was added to growing cultures and 2DG had no effect on stationary-phase cultures. 2DG-treated cells had weakened cell walls that were more susceptible to degradation by

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Abbreviations: 2DG, 2-deoxyglucose; LTA, lipoteichoic acid; PTS, phosphotransferase system.

lysozyme and it appeared that 2DG was preventing inactivation of cell-wall-hydrolysing enzymes (Russell & Wells, 1997).

Wild-type *S. bovis* cells have a glucose phosphotransferase (PTS) system that can also phosphorylate 2DG (Martin & Russell, 1986) and 2DG 6-phosphate can be dephosphorylated by a phosphatase (Cook *et al.*, 1995). This cycle of phosphorylation and dephosphorylation can slow growth and de-energize the cell (Thompson, 1987). *S. bovis* also has a facilitated diffusion system for glucose, allowing mutants lacking the glucose-PTS to still grow rapidly on glucose (Russell, 1990; Cook & Russell, 1994).

In many Gram-positive organisms, lipoteichoic acids (LTAs) have been shown to inactivate cell-wall-degrading enzymes and LTAs are often glycosylated by UDP-glucose that arises from glucose 1-phosphate (Fischer, 1988, 1994). *Saccharomyces* can produce 2DG 1-phosphate and UDP-2DG (Biely *et al.*, 1971), but synthesis of these phosphorylated sugars has not been investigated in prokaryotes. However, because group D streptococcal LTAs contain glucose residues linked via an α -1,2 bond (kojibiose and kojitrise) (Wicken & Baddiley, 1963; Wicken & Knox, 1975), we hypothesized that 2DG-induced lysis might be mediated via synthesis of abnormal LTAs.

The following experiments sought to (1) select and characterize 2DG-resistant mutants, (2) examine the relationship between sugar phosphorylation and 2DG-induced lysis, (3) determine if LTAs could counteract 2DG-induced lysis and (4) monitor the possible incorporation of 2DG into LTAs of *S. bovis*.

METHODS

Cell growth. *Streptococcus bovis* JB1 was routinely grown under O₂-free CO₂ at 39 °C in basal medium containing (l⁻¹): 2 g glucose, 292 mg K₂HPO₄, 292 mg KH₂PO₄, 480 mg (NH₄)₂SO₄, 480 mg NaCl, 100 mg MgSO₄·7H₂O, 64 mg CaCl₂·2H₂O, 500 mg cysteine HCl, 1 g Trypticase (BBL Microbiology Systems), 4 g Na₂CO₃ and 0.5 g yeast extract. The medium was adjusted to pH 6.7 and the final pH was never less than 6.5. When indicated, 2-deoxy-D-glucose (2DG) was added prior to inoculation at a concentration of 2 mg ml⁻¹. Growth was monitored via changes in OD₆₀₀ in 18 × 150 mm tubes that were sealed with butyl rubber stoppers.

Lysis assays. Lysis was typically monitored by following decreases in optical density after *S. bovis* cultures had reached stationary phase. Proteinase K, when indicated, was added directly to stationary-phase cultures at a final concentration of 1 mg ml⁻¹. When cell extracts or semi-purified LTA was added, additions were made at the onset of stationary phase, at a concentration of 75 µg carbohydrate (mg cell protein)⁻¹ unless otherwise indicated.

Lysis was also monitored by suspending *Micrococcus lysodeikticus* (*Micrococcus luteus*) cells (ATCC 4698; Sigma) in supernatants from lysed *S. bovis* cultures and measuring changes in optical density. *S. bovis* Lys⁺ cultures were grown in the presence of 2DG, allowed to lyse for 48 h and centrifuged anoxically (15 min, 3500 g) to remove residual cells. After supernatants were transferred to anoxic tubes, *M.*

lysodeikticus cells were added from a concentrated stock to a final OD₆₀₀ of 1.0 (approx. 150 mg cell protein l⁻¹) and tubes were incubated at 39 °C. When cell extract or semi-purified LTA was added [75 µg carbohydrate (mg cell protein)⁻¹], tubes with an equivalent amount of sodium phosphate buffer served as a control.

For cell wall lysis assays, cell walls were isolated essentially according to Blackman *et al.* (1998). Cells grown in the presence or absence of 2DG were harvested and washed three times in ddH₂O + 0.5 mM PMSF (10000 g, 5 min, 4 °C). Concentrated cells were subjected to two passes through a pre-cooled French pressure cell (138000 kPa), centrifuged for 15 min (3000 g, 4 °C) (to pellet unbroken cells) and the top two-thirds of this suspension was carefully removed for pelleting of cell walls (25000 g, 4 °C). Walls were washed in 100 mM NaCl twice to remove loosely associated proteins and each time care was taken to not resuspend the more solid pellet consisting of contaminating cells and membrane vesicles. Walls were washed twice in 50 mM Tris/HCl + 1 mM MgCl₂ buffer, pH 6.5, and resuspended to a final OD₆₀₀ of approximately 10. Lysis was immediately assayed by diluting walls in 50 mM Tris/HCl + 1 mM MgCl₂ + 1 mM dithiothreitol, pH 6.5, and monitoring the decrease in OD₆₀₀ (39 °C). Rates of lysis were lower in the absence of dithiothreitol. The purity of these preparations (presence of walls and not vesicles) was confirmed by addition of lysozyme, which caused a rapid decrease in the OD₆₀₀ to less than 5% of original levels. Some walls were also assayed after one freeze-thaw cycle (storage at -20 °C). Further freeze-thaw cycles reduced lytic activity and altered protein profiles as visualized by SDS-PAGE (see below). Proteinase K, when indicated, was added at a final concentration of 1 mg ml⁻¹. When cell extract or semi-purified LTA was added, 200 µg carbohydrate was added per OD₆₀₀ unit of cell walls.

Selection of lysis-resistant and glucose-PTS-deficient phenotypes. 2DG-treated (2 mg glucose ml⁻¹ and 2 mg 2DG ml⁻¹) *S. bovis* JB1 cultures were transferred at least seven times (<1% inoculum) every 6 or 72 h. As transport of 2DG via the glucose-PTS slows growth, 6 h transfers selected for cells able to grow rapidly due to a lack of the glucose/2DG-PTS. Longer (72 h) transfers selected for lysis-resistant cells, while retaining pressure to keep the PTS due to its role in recovery from starvation. Once cultures yielded a desired phenotype (PTS⁻ or Lys⁻), they were streaked onto an agar plate containing basal medium and 10 mg ml⁻¹ each of glucose and 2DG. A small number of individual colonies were restreaked, tested for PTS activity and lysis sensitivity and one of each phenotype (PTS⁻ or Lys⁻) was retained. The PTS⁻ culture (Lys⁺ PTS⁻) was subjected to a series of 72-h transfer intervals to obtain the other phenotype (Lys⁻ PTS⁻). The stability (lack of reversion) of each isolate was monitored by transferring cultures without 2DG every 12 h and testing each successive culture for 2DG-dependent lysis and PTS activity.

PTS assays. PEP-dependent sugar phosphorylation was assayed enzymically by a method that measures pyruvate production via lactate dehydrogenase (Martin & Russell, 1986). Cells were harvested (10 ml) during exponential growth by centrifugation (10000 g, 10 min, 4 °C) and washed in 100 mM sodium/potassium phosphate buffer (25 mM Na⁺, 75 mM K⁺, 5 mM MgCl₂, 1 mM dithiothreitol, pH 7.2). Cells were resuspended in the same buffer and stored on ice. Cells (250 µl) were treated with 8 µl toluene/ethanol mixture (1:9, v/v), subjected to vigorous agitation for 1 min and used immediately. All PTS assays were performed at 39 °C, corrections were made for non-specific NADH oxidase

activity and specific activities were determined under enzyme-limiting conditions (protein concentration versus activity was linear). Each assay typically contained (ml^{-1}): 10 μl of the toluenized cells (approx. 10 μg protein), 9 units L-lactic dehydrogenase, 0.84 μmol DTT, 4.2 μmol MgCl_2 , 8.6 μmol PEP, 0.17 μmol NADH, 1.6 μmol sugar, 75 μmol potassium phosphate and 25 μmol sodium phosphate.

Phosphorylation of 2DG. Cultures (40 ml) were washed as described for the PTS assay and resuspended in 2 ml sodium/potassium phosphate buffer (approx. 6 mg protein ml^{-1}). Concentrated cells were toluenized with 30 μl toluene/ethanol (1:9, v/v) for 1 min and immediately divided into two tubes containing 2DG (10 mM) plus either ATP or PEP (10 mM). Cells were incubated for 30 min at 39 °C and centrifuged (10000 g, 10 min). The supernatant was frozen and analysed via TLC (see below).

LTA extracts. LTA was extracted from cells using hot aqueous phenol (Sijtsma *et al.*, 1990; Hogg *et al.*, 1997). Cultures (4 l) were centrifuged (10000 g, 15 min, 25 °C), washed in phosphate buffer (10 mM Na_2HPO_4 , 1 mM MgCl_2 , pH 7.5) and concentrated to a final concentration of approx. 0.4 g cells ml^{-1} . The cell suspension was stirred with an equal amount of pre-warmed 85% phenol (65 °C, 90 min) and centrifuged (10000 g, 20 min, 25 °C) to obtain phase separation. The water layer was removed, extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v), centrifuged again and the water layer retained. Crude extract (or phosphate buffer processed in a similar fashion with phenol and chloroform as a control) was added to stationary-phase cultures (0.05–0.5 ml into 10 ml) and 2DG-induced lysis was monitored as described above. The extract was further purified by treatment with RNase and DNase (20 μg ml^{-1} each, 30 °C, 8 h), extracted twice with cold phenol/chloroform/ethanol (25:24:1, by vol.) and dialysed 24 h (3000 kDa cut-off) against 10000 volumes of Tris buffer (10 mM Tris/HCl, 1 mM MgCl_2 , pH 7.5, 4 °C) to remove solvents and nucleic acid fragments. Assuming 2 μmol glucose equivalents (mg LTA^{-1}) (Kessler *et al.*, 1983), extractions from all cell types yielded approximately 2–4 mg LTA (g cells) $^{-1}$.

TLC. Equal amounts of anthrone-reactive material or sugar standards (typically 1–3 μg hexose equivalent) were applied to spots 1 cm apart on TLC plates (Merck Art. 5737 silica gel 60/kieselguhr F₂₅₄ pre-coated, layer thickness 0.25 mm) and separated by immersing the bottom 1 cm of the plate in n-propanol/ethyl acetate/water (7:1:4, by vol.). Dried plates were sprayed with an anisaldehyde-based reagent (27 ml ethanol, 0.3 ml acetic acid, 1.5 ml sulfuric acid, 1.5 ml *p*-anisaldehyde) and placed in a 110 °C oven for 5 min. Under these conditions and at the concentrations typically used, standards of the sugars glucose, fructose, galactose, glucosamine, ribose, xylose, arabinose and 2DG all stained as unique colours against a pink background, while maltose, cellobiose, lactose and melibiose stained similar to glucose. To confirm the presence of 2DG in extracts, duplicate plates were stained with an anthrone-based stain (prepared by dissolving 0.3 g anthrone in 10 ml acetic acid and mixing with 20 ml ethanol, 3 ml phosphoric acid and 1 ml water) and placed in a 110 °C oven for 5 min. Under these conditions and at the concentrations typically used, no colour was observed for glucose, galactose, glucosamine, maltose, cellobiose, lactose or melibiose, but 2DG stained dark blue. Fructose, ribose, xylose and arabinose stained shades of yellow.

TLC was also used to monitor glucose and 2DG phosphorylation. Supernatants from toluenized cells incubated

with 2DG and either ATP or PEP as the phosphoryl donor were applied to similar TLC plates and separated using the same solvent mixture. Under these conditions, glucose could be separated from UDP-glucose and glucose 6-phosphate migrated similar to glucose 1-phosphate. Thus, the production of 2DG phosphates could be monitored, but we could not determine if the product was 2DG 1-phosphate or 2DG 6-phosphate.

Other analyses. Cell wall samples for SDS-PAGE were added directly to sample buffer (final concentration 1% SDS, 5% glycerol, 10 mM Tris/HCl, 10 mM DTT), boiled for 3 min, centrifuged at 14000 g for 15 min to remove cell walls and subjected to electrophoresis on 12% polyacrylamide gels under denaturing conditions according to Laemmli (1970). Carbohydrate in cell extracts was assayed by the anthrone method (Bailey, 1958) using glucose as standard and nucleic acids did not interfere with this assay. Protein was determined using the NanoOrange reagent (Molecular Probes).

RESULTS

Lysis and selection of PTS and lysis-deficient phenotypes

When *S. bovis* JB1 was incubated with glucose and 2DG, the cells grew nearly as fast as untreated controls, but lysis was observed after the cultures reached stationary phase (>90% lysis after 48 h). Significant lysis was only observed if 2DG was present during the entire growth phase. If 2DG was added in late exponential phase or during stationary phase, little lysis was observed (Fig. 1). Proteinase K accelerated the lysis of *S. bovis* cultures grown with 2DG (80% lysis after 24 h), but it had no effect on glucose-grown cultures (data not shown). Cell walls prepared from wild-type cultures grown with glucose were stable for long periods of time

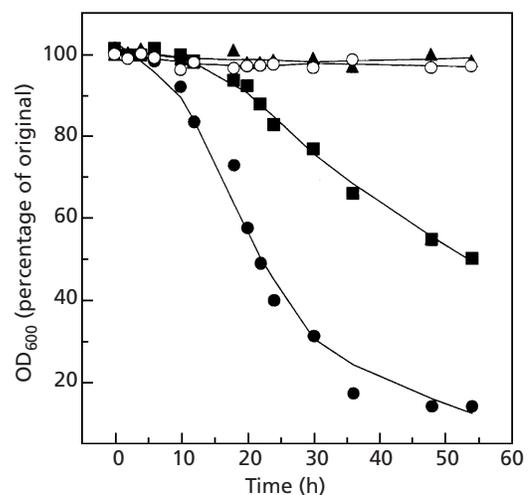


Fig. 1. The effect of 2DG (2 mg ml^{-1}) addition on the optical density of *S. bovis* cells grown with glucose (2 mg ml^{-1}). 2DG was added at the onset of stationary phase (○), mid-exponential phase (■) or at inoculation (●). Controls with no 2DG (▲) are also shown.

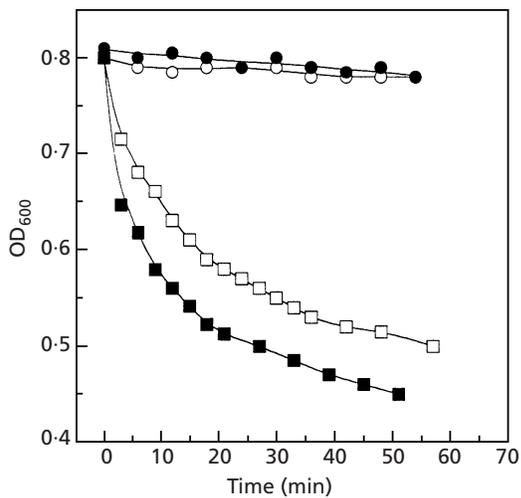


Fig. 2. The optical density of *S. bovis* cell walls that were isolated from glucose-grown (○, ●) and glucose + 2DG-grown (□, ■) cultures and incubated in Tris buffer (50 mM, pH 7.0, 1 mM MgCl₂, 1 mM dithiothreitol, 39 °C). Filled symbols show cell walls that were treated with proteinase K (1 mg ml⁻¹).

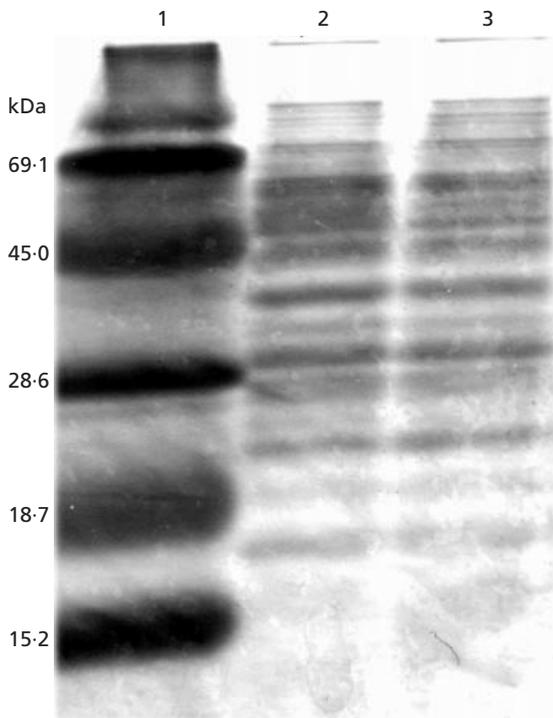


Fig. 3. SDS-PAGE of proteins associated with cell walls isolated from *S. bovis*. Lanes: 1, molecular mass standard; 2, cell walls from cultures grown with glucose; 3, cell walls from cultures grown with glucose plus 2DG.

at 39 °C (Fig. 2). Cell walls prepared from cultures grown with glucose and 2DG decayed rapidly if they were incubated at 39 °C and this initial decay could also

be accelerated by proteinase K. PAGE gels indicated that cells grown with glucose or glucose plus 2DG had similar cell-wall-associated proteins (Fig. 3).

When wild-type *S. bovis* JB1 (Lys⁺ PTS⁺) was transferred every 6 h with glucose and 2DG, cells lost their glucose- and 2DG-PTS activities, but 2DG-dependent lysis was still observed (Lys⁺ PTS⁻, Table 1). When the Lys⁺ PTS⁻ culture was transferred with glucose and 2DG every 72 h, a lysis-resistant, PTS⁻ phenotype emerged (Lys⁻ PTS⁻). Wild-type cells (Lys⁺ PTS⁺) that were grown with glucose and 2DG every 72 h also became lysis-resistant, but PTS activity was retained (Lys⁻ PTS⁺). Further work was based on cultures obtained via colony re-isolation from each culture (Lys⁺ PTS⁺, Lys⁻ PTS⁺, Lys⁺ PTS⁻, Lys⁻ PTS⁻). These cultures did not revert to the wild-type phenotype when cultures were allowed to grow for 50 generations without selective pressure.

Toluene-permeabilized wild-type and Lys⁻ PTS⁺ cells phosphorylated 2DG when PEP was present and a phosphorylated 2DG derivative could be detected on TLC plates sprayed with anisaldehyde stain (Fig. 4). Virtually no PEP-dependent 2DG phosphorylation was detected with the Lys⁺ PTS⁻ and Lys⁻ PTS⁻ cells. When ATP was added as the phosphoryl donor, the wild-type, Lys⁻ PTS⁺ and Lys⁺ PTS⁻ cells produced a 2DG phosphate derivative, but the Lys⁻ PTS⁻ isolate produced less of this product. No 2DG phosphorylation was detected from any cell type with GTP, ITP or UTP as the donor.

LTA extract

When wild-type cells (grown without 2DG) were treated with 85% hot phenol, the water soluble extract prevented 2DG-dependent lysis of wild-type cells (Fig. 5). An extract from wild-type cells grown with glucose and 2DG was approximately 100-fold less effective than extract prepared from cells grown with only glucose (Fig. 6). The crude extracts had an abundance of anthrone-reactive carbohydrate (>500 mg l⁻¹), nucleic acids (>100 µg ml⁻¹) and small amounts of protein (<50 µg ml⁻¹), but nuclease treatments, phenol/chloroform precipitations and dialysis removed additional protein (<20 µg ml⁻¹) and nucleic acids (<50 µg ml⁻¹), while the anthrone-reactive material remained. The dialysed extract from glucose-grown cells was as effective in preventing the 2DG-dependent lysis of Lys⁺ strains as the crude extract, and dialysed extracts of lysis-sensitive strains (Lys⁺) grown with 2DG still did not prevent wild-type lysis.

Dialysed extracts prevented 2DG-dependent lysis of *S. bovis* cells that were treated with proteinase K and these extracts could even stabilize cells that had already initiated an increased lysis rate (Fig. 7). Dialysed extracts did not affect the initial degradation rate of cell walls prepared from 2DG-grown cultures and the difference in cell wall autolysis was not nearly so dramatic after 48 h as seen in whole cells treated with similar extracts

Table 1. PTS activities and lysis sensitivities of four different phenotypes of *S. bovis*

Data are the means of three determinations \pm SD.

Phenotype	Glucose-PTS*	2DG-PTS*	Percentage lysis after 48 h†
Wild-type (<i>Lys</i> ⁺ <i>PTS</i> ⁺)	668.7 \pm 95.1	696.5 \pm 43.6	92.2 \pm 4.2
<i>Lys</i> ⁺ <i>PTS</i> ⁻	46.3 \pm 10.4	22.5 \pm 12.1	90.3 \pm 3.1
<i>Lys</i> ⁻ <i>PTS</i> ⁺	329.6 \pm 21.6	366.2 \pm 45.1	5.7 \pm 5.1
<i>Lys</i> ⁻ <i>PTS</i> ⁻	20.8 \pm 11.2	ND	8.3 \pm 3.1

* Expressed as nmol NADH oxidized (mg protein)⁻¹ min⁻¹ in the presence of the sugar indicated, corrected for non-specific NADH oxidase activity. ND, Not detected.

† Percentage decrease in OD₆₀₀ after 48 h compared to that at the end of exponential growth phase.

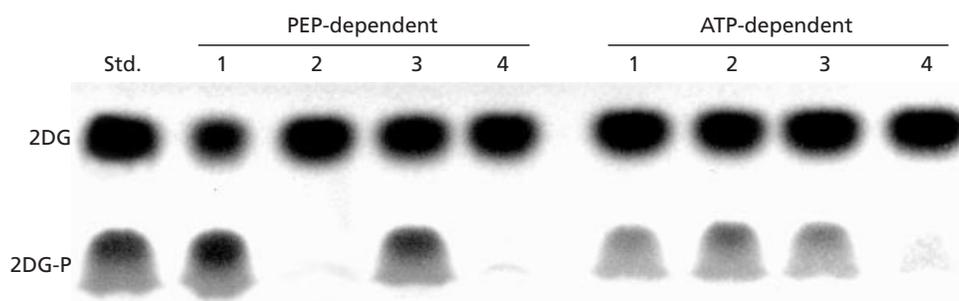


Fig. 4. TLC of 2DG and 2DG phosphate (2DG-P) produced by *S. bovis* cells. Toluene-treated cells were incubated with 2DG and either PEP or ATP as a phosphoryl donor. Lanes: 1, wild-type cells; 2, *Lys*⁺ *PTS*⁻; 3, *Lys*⁻ *PTS*⁺; 4, *Lys*⁻ *PTS*⁻. The standard (Std.) was a mixture of 2DG and 2DG 6-phosphate.

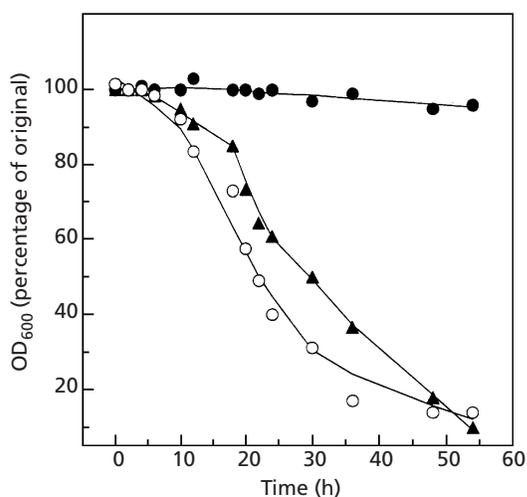


Fig. 5. The effect of semi-purified LTA extracts on the optical density of stationary phase *S. bovis* cells grown with 2DG. Extracts were derived from wild-type cells grown on glucose (●) or wild-type cells grown with glucose and 2DG (▲). Controls with no extract (○) are also shown. The extracts were added to achieve 75 μ g carbohydrate per mg cell protein.

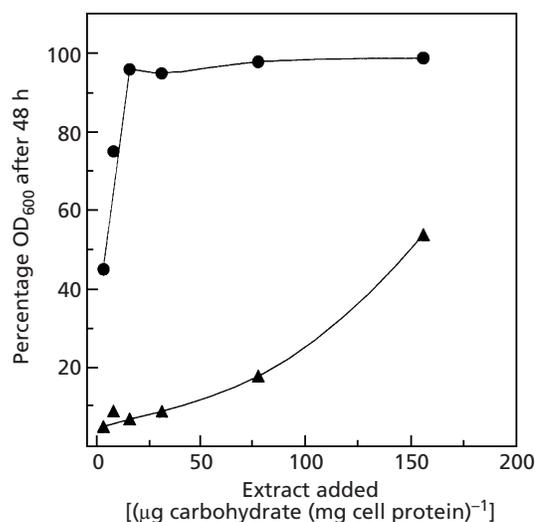


Fig. 6. The effect of semi-purified LTA extracts on the lysis of stationary phase *S. bovis* cells grown with 2DG. Extracts were derived from wild-type cells grown on glucose (●) or wild-type cells grown with glucose and 2DG (▲).

(Table 2). *M. lysodeikticus* cells that were incubated in media with or without 2DG at 39 °C did not lyse and supernatant from 48 h wild-type *S. bovis* cultures grown

on glucose had little effect on these cells (data not shown). Supernatants from *S. bovis* cultures grown on glucose and 2DG promoted the lysis of *M. lysodeikticus*

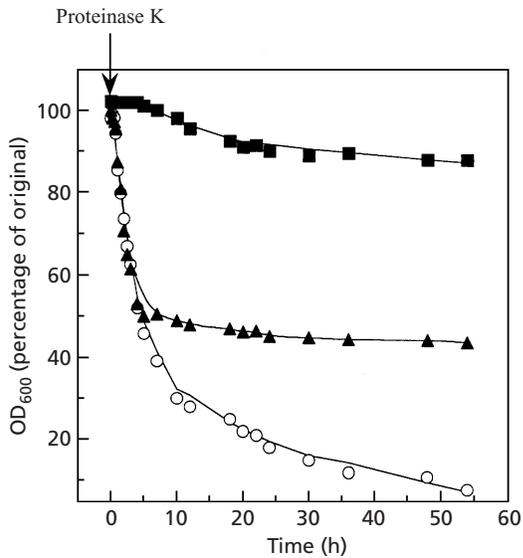


Fig. 7. The effect of semi-purified LTA extracts on the optical density of stationary phase *S. bovis* cells grown with 2DG and treated with proteinase K. Extracts were added at 0 (■) or 5 (▲) h after proteinase K addition. Controls with no extract (○) are also shown. Extracts [75 µg carbohydrate (mg cell protein)⁻¹] were derived from wild-type cells grown on glucose alone. Cells which did not receive extract or proteinase K are shown Fig. 1 and Fig. 5.

Table 2. Effect of semi-purified LTA extract from glucose-grown cells on lysis of *S. bovis* cells, isolated cell walls and *M. lysodeikticus* cells

Data are the means of two determinations ± SD.

Treatment	Percentage OD ₆₀₀ remaining after 48 h		
	<i>S. bovis</i> *	Cell wall†	<i>M. lysodeikticus</i> ‡
None	8.4 ± 3.1	33.6 ± 5.2	45.3 ± 4.8
+ LTA extract	94.1 ± 4.2	40.2 ± 6.3	96.3 ± 3.3

* *S. bovis* cells grown with glucose + 2DG.

† Cell walls isolated from 2DG-grown cultures of *S. bovis*.

‡ *M. lysodeikticus* cells + supernatant from 2DG-grown *S. bovis* cultures.

cells and this lysis could also be inhibited by the dialysed extract (Table 2).

Incorporation of 2DG into LTA

Anisaldehyde stain was able to detect both 2DG and glucose on TLC plates, but these compounds migrated and stained differently (blue vs green) (Fig. 8a, lane 1). The ability of TLC to differentiate these sugars could be enhanced by the use of an anthrone-based stain. The anthrone stain reacted strongly with 2DG (Fig. 8b, lane 1), but did not produce any colour with glucose. When

phenol extracts from wild-type *S. bovis* grown on glucose were applied to the TLC plates and stained with anisaldehyde, several sugar-containing bands were observed that stained similar to glucose (Fig. 8a, lane 2). These bands were not visible if an anthrone-based strain was used (Fig. 8b, lane 2). When wild-type cells were grown with glucose and 2DG, the major carbohydrate-containing band migrated faster (Fig. 8a, lane 3) and stained blue rather than green (indicative of 2DG). This band also reacted strongly and stained similar to 2DG when the anthrone-based stain was used (Fig. 8b, lane 3). Sugar-containing substances from the lysis-sensitive, PTS⁻ isolate (Lys⁺ PTS⁻) migrated the same and had the same colour as those from wild-type cells (Fig. 8a, b, lanes 4). The lysis-resistant (Lys⁻ PTS⁺ and Lys⁻ PTS⁻) extracts had anisaldehyde-reactive carbohydrate that stained positive for glucose, but reacted poorly with the 2DG-detecting anthrone-based stain (Fig. 8a, b, lanes 5 and 6).

DISCUSSION

The rumen is an ideal habitat for the growth of anaerobic bacteria, but ¹⁵N labelling experiments indicate that as much as 33% of the microbial protein turns over before it leaves the rumen (Nolan, 1975). Ruminal protozoa can engulf and digest some bacteria, but Krebs *et al.* (1989) demonstrated that microbial turnover was nearly as great in protozoa-free sheep. The rumen environment also contains bacteriophage and aneroplasmas, which could contribute to bacterial lysis, but pure cultures of many rumen species also lyse rapidly (Wells & Russell, 1996b). *S. bovis* JB1 is a relatively stable bacterium, but previous work indicated that it would lyse if 2DG was included in the growth medium (Russell & Wells, 1997). This observation made it possible to study an aspect of lysis regulation in *S. bovis*.

Bacterial stability is bolstered by peptidoglycan, but peptidoglycan-degrading enzymes are needed for many important processes, including cell wall assembly (Shockman & Barrett, 1983), cell separation (Foster, 1995), flagellar synthesis (Pooley & Karamate, 1984) and sporulation (Foster, 1992). Some of these enzymes can cause the lysis of intact cells (Shockman & Barrett, 1983) and these 'autolysins' must be carefully regulated during growth and after the cells have reached stationary phase. *Bacillus* has autolysins that are regulated at the transcriptional level and are only transcribed during sporulation (Foster, 1992), but post-translational regulation is also commonly observed (Jolliffe *et al.*, 1980; Buist *et al.*, 1988).

Autolysins of bacteria are strongly associated with the cell wall, where they can be further regulated by proteolytic inactivation (Rogers *et al.*, 1984; Wells & Russell, 1996a; Buist *et al.*, 1988). Conversely, some autolysins are secreted as proenzymes and can be activated by proteinases (Shockman *et al.*, 1967; Kariyama & Shockman, 1992). PAGE gels indicated that *S. bovis* cultures grown with or without 2DG had

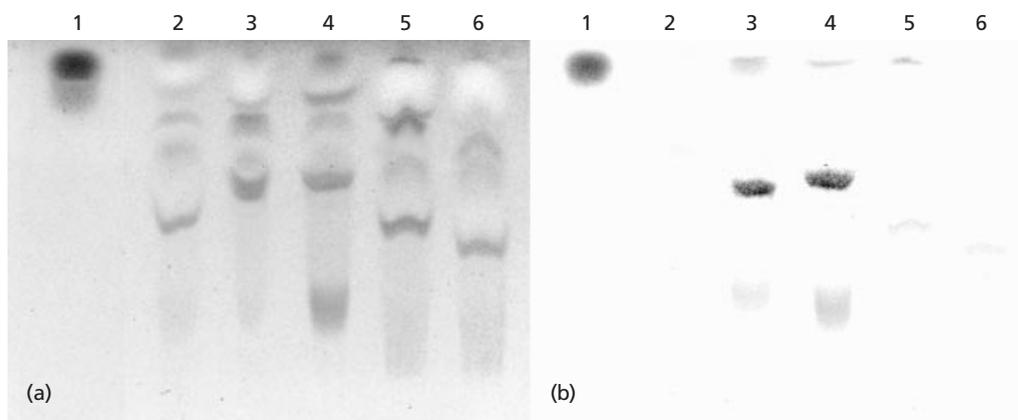


Fig. 8. TLC of LTA extracts. Identical plates were sprayed with an anisaldehyde stain (a) or an anthrone-based stain (b). Lanes: 1, a standard mixture of 2DG (upper spot) and glucose (lower); 2, LTA extract from wild-type cells grown only with glucose; 3, extract from wild-type cells grown with glucose and 2DG; 4, extract from $\text{Lys}^+ \text{PTS}^-$ grown with glucose and 2DG; 5, extract from $\text{Lys}^- \text{PTS}^+$ grown with glucose and 2DG; 6, extract from $\text{Lys}^- \text{PTS}^-$ grown with glucose and 2DG.

similar cell-wall-associated proteins and the 2DG-dependent lysis of *S. bovis* could be accelerated by proteinases. Based on these observations, 2DG-grown cells seemed to have the same autolysins as glucose-grown cells and growth with 2DG was interfering with autolytic regulation.

Previous workers demonstrated that autolytic inactivation can be affected by other cell wall components, such as LTA (Höltje & Tomasz, 1975; Diederick Meyer & Wouters, 1987; Cleveland *et al.*, 1976a; Roger *et al.*, 1984; Fischer, 1988; Jolliffe *et al.*, 1980). Most Gram-positive species have LTA in their cell wall (Fischer, 1994) and in streptococci, LTAs are commonly found as diacylglycerolipid-anchored chains of 1,3-linked poly-(glycerolphosphate) (Fischer, 1988). The C-2 of each glycerol residue in the LTA backbone is often substituted by sugars and/or D-alanine esters (Fischer *et al.*, 1981). LTA has been shown to inhibit autolysins from bacilli (Rogers *et al.*, 1984; Wecke *et al.*, 1997), enterococci (Cleveland *et al.*, 1976a), streptococci (Höltje & Tomasz, 1975) and staphylococci (Fischer *et al.*, 1981), and in some cases, the degree of inhibition has been correlated with alterations at the C-2 position of the LTA glycerolphosphate chain (Fischer *et al.*, 1981; Wecke *et al.*, 1997).

Organisms that react with the Lancefield group D antibody have LTAs substituted with either kojibiose or kojitriose, an α -1,2 linked glucose di- or trisaccharide (Wicken & Knox, 1975; Kessler *et al.*, 1984). Because it is chemically impossible to synthesize kojibiose from 2DG, it appeared that the 2DG-dependent lysis of *S. bovis* (a group D streptococcus) was being mediated via an alteration in LTA substitution which allowed increased autolysin activity. This idea was supported by several observations: (1) 2DG was detected in LTA extracts from wild-type cells grown with 2DG, (2) 2DG was not detected in LTA extracts of lysis-resistant isolates, even if 2DG was added to the growth medium, (3) glucose-containing LTA extracts could prevent 2DG-

dependent lysis in *S. bovis*, even when it was stimulated by proteinase K and (4) 2DG-containing LTA extracts could not prevent 2DG-dependent lysis. LTA inhibited the lysis of *M. lysodeikticus* cells that were suspended in supernatants from 2DG-treated *S. bovis* cultures, but LTA was not able to reduce the degradation of *S. bovis* cell walls that were obtained from 2DG-treated cultures. These latter findings indicate that LTA is effective in intact cells, but this effect may be lost when cells are sheared and cell walls repeatedly washed in sodium chloride.

Because most bacterial glucose kinases cannot recognize 2DG, net 2DG phosphorylation has often been used as an index of PTS activity (Romano *et al.*, 1979). TLC confirmed that PTS^- isolates had reduced PEP-dependent 2DG phosphorylation, but significant ATP-dependent 2DG phosphorylation was still detected in wild-type *S. bovis* and one of the PTS^- isolates. The idea that ATP-dependent 2DG phosphorylation might play a role in 2DG-dependent lysis was supported by the observation that one of the lysis-resistant isolates ($\text{Lys}^- \text{PTS}^-$) had significantly less activity than wild-type cells. The other lysis-resistant isolate ($\text{Lys}^- \text{PTS}^+$) retained PTS activity as well as ATP-dependent 2DG phosphorylation and this indicated that accumulation of intracellular 2DG phosphate alone does not trigger lysis (Prasad & Freese, 1974). This $\text{Lys}^- \text{PTS}^+$ isolate still produced normal glucose-containing LTA, even if 2DG was present, indicating that some later step in LTA synthesis had been modified to exclude 2DG.

Autolytic enzymes can also be inhibited by amphiphiles such as cardiolipin and phosphatidyl glycerol (Höltje & Tomasz, 1975; Cleveland *et al.*, 1976b; Diederick Meyer & Wouters, 1987), bringing the specificity of LTAs as autolysin inhibitors into question. Some LTAs have alanine residues extending from the glycerolphosphate backbone or carbohydrate side chains and this substitution decreases the ability of LTAs to inhibit autolysins from *Staphylococcus aureus* and *Bacillus*

subtilis (Fischer *et al.*, 1981; Wecke *et al.* 1997). These labile alanine residues are reportedly lost under the conditions used in our extraction procedures (Fischer & Koch, 1981), but extracts from *S. bovis* maintained similar activity at all stages of purification, suggesting that alanine substitution was not responsible for differences in autolysin–LTA interactions in this organism. Glycosyl substitution, another structural feature that could confer specificity, did not significantly affect autolysis in *S. aureus* (Fischer *et al.* 1981). However, increased glucose substitution of LTA (caused by chloramphenicol treatment) has been correlated with a decrease in the autolysis of *Enterococcus faecalis* (Pooley & Shockman, 1970; Kessler *et al.*, 1983). When *S. bovis* was grown in 2DG-containing medium, it incorporated 2cDG into its LTA and this LTA lost virtually all of its ability to inhibit autolysis of whole cells. These results and the observation that isolates resistant to lysis did not incorporate 2DG into their LTA, indicate that the carbohydrate side chain of LTA may be an important feature of autolysin regulation in *S. bovis*.

DISCLAIMER

Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product and the use of the name by the USDA implies no approval of the product and exclusion of others that may be suitable.

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